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*T. Lawrence*

T. LAWRENCE

Certifying Officer

## PROVISIONAL APPLICATION COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION under 37 CFR 1.53 (b)(2).

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LAST NAME	FIRST NAME	MIDDLE INITIAL	RESIDENCE (CITY AND EITHER STATE OR FOREIGN COUNTRY)		
HERSCOVICS	Annette	A.	4837 Hutchison St., #5, Montréal, Québec, Canada H2V 4A4		
TREMBLAY	Linda	O.	5156 Pierre Tétreault, Montréal, Québec, Canada H1K 2Y8		
TITLE OF THE INVENTION (280 characters max)					
α1,2-MANNOSIDASE AND THERAPEUTICAL USES THEREOF					
CORRESPONDENCE ADDRESS					
France Côté SWABEY OGILVY RENAULT 1981 McGill College Avenue, Suite 1600, Montréal					
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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

- ☒ No
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Respectfully submitted,

SIGNATURE

*France Côté*

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TYPED or PRINTED NAME

France Côté

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☐ Additional inventors are being named on separately numbered sheets attached hereto.

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## $\alpha$ 1,2-MANNOSIDASE AND THERAPEUTICAL USES THEREOF

### BACKGROUND OF THE INVENTION

#### (a) Field of the Invention

5           The invention relates to a novel  $\alpha$ 1,2-mannosidase and its  
therapeutic uses thereof in the treatment of genetic diseases.

#### (b) Description of Prior Art

$\alpha$ 1,2-Mannosidases are essential for hybrid and complex N-  
glycan biosynthesis in mammalian cells (Herscovics, 1999). Following the  
10       removal of the glucose residues from the  $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$  precursor  
structure attached to nascent glycoproteins, ER and Golgi  $\alpha$ 1,2-  
mannosidases catalyse the trimming of the four  $\alpha$ 1,2-linked mannose  
residues. The subsequent action of GlcNAc transferase I initiates complex  
chain formation and yields the substrate for Golgi  $\alpha$ -mannosidase II which  
15       trims the terminal  $\alpha$ 1,3- and  $\alpha$ 1,6-mannose residues. In some tissues a  
distinct  $\alpha$ -mannosidase trims  $\text{Man}_5\text{GlcNAc}_2$  to  $\text{Man}_3\text{GlcNAc}_2$  prior to the  
action of GlcNAc transferase I. Thereafter the N-glycan structure is further  
elaborated by Golgi glycosyltransferases.

$\alpha$ -Mannosidases have been classified into two groups based on  
20       amino acid sequence homology and on biochemical properties. Class I  $\alpha$ -  
mannosidases specifically hydrolyze  $\alpha$ 1,2-linked mannose residues, and  
do not cleave substrates such as p-nitrophenyl- $\alpha$ -D-mannopyranoside.  
They require calcium for activity and are inhibited by 1-  
deoxymannojirimycin and kifunensine, but not by swainsonine. In contrast,  
25       Class II  $\alpha$ -mannosidases can cleave  $\alpha$ 1,2-,  $\alpha$ 1,3- and  $\alpha$ 1,6-linked  
mannose residues as well as p-nitrophenyl- $\alpha$ -D-mannopyranoside and are  
inhibited by swainsonine, but not by 1-deoxymannojirimycin.

          Although several mammalian  $\alpha$ 1,2-mannosidases that can  
remove up to four  $\alpha$ 1,2-mannose residues have been purified and cloned,  
30       there is significant biochemical evidence for the existence of highly

specific mammalian enzymes that trim  $\text{Man}_9\text{GlcNAc}_2$  to  $\text{Man}_8\text{GlcNAc}_2$  isomer B, the form lacking the middle-arm terminal  $\alpha 1,2$ -mannose, but mammalian enzymes with this specificity have not yet been purified or cloned. A mammalian ER  $\alpha 1,2$ -mannosidase that forms  $\text{Man}_8\text{GlcNAc}_2$  isomer B and is not sensitive to 1-deoxymannojirimycin was described in intact UT-1 cells and in rat hepatocytes whereas distinct 1-deoxymannojirimycin-sensitive  $\alpha 1,2$ -mannosidase activity that processes  $\text{Man}_9\text{GlcNAc}_2$  to  $\text{Man}_8\text{GlcNAc}_2$  isomer B was observed in the ER of intact COS cells and in ER and in Golgi rat liver membrane preparations. Up to now the yeast ER processing  $\alpha 1,2$ -mannosidase is the only enzyme purified (Jelinek-Kelly and Herscovics, 1988) and cloned (Camirand *et al.*, 1991) that specifically trims  $\text{Man}_9\text{GlcNAc}_2$  to  $\text{Man}_8\text{GlcNAc}_2$  isomer B.

It would be highly desirable to be provided with the isolation, expression, and properties of a novel human cDNA encoding a Class I  $\alpha 1,2$ -mannosidase that specifically converts  $\text{Man}_9\text{GlcNAc}$  to  $\text{Man}_8\text{GlcNAc}$  isomer B.

#### **SUMMARY OF THE INVENTION**

One aim of the present invention is to provide the isolation, expression, and properties of a novel human cDNA encoding a Class I  $\alpha 1,2$ -mannosidase that specifically converts  $\text{Man}_9\text{GlcNAc}$  to  $\text{Man}_8\text{GlcNAc}$  isomer B.

In accordance with the present invention there is provided a human  $\alpha 1,2$ -mannosidase enzyme for specifically converting  $\text{Man}_9\text{GlcNAc}$  to  $\text{Man}_8\text{GlcNAc}$  isomer B in degradation mechanism of misfolded proteins, wherein the enzyme has the characteristics of an enzyme encoded by a cDNA sequence set forth in Fig. 1.

In accordance with the present invention there is also provided the tools to develop specific agonist or antagonist of this particular  $\alpha 1,2$ -mannosidase that would not affect the other mannosidases.

The agonist or antagonist may provide for activating or inhibiting for a transient period of time. For example, such an antagonist may be

inhibiting the enzyme for a transient period of time, meanwhile preventing misfolded glycoproteins from being degraded.

In accordance with the present invention there is also provided the potential for a method for the treatment of genetic diseases causing misfolding of proteins in a patient, which comprises administering an antagonist of  $\alpha$ 1,2-mannosidase enzyme for transiently inhibiting the enzyme, thereby prevent misfolded glycoproteins from degradation.

For example, such a genetic disease includes, without limitation, cystic fibrosis, emphysema, among others.

The misfolded protein for cystic fibrosis is, for example, cystic fibrosis transmembrane conductance regulator (CFTR) (Ward et al., 1995).

The misfolded protein for emphysema is, for example, alpha1 antitrypsin.

For the purpose of the present invention the following abbreviations are defined below.

ER	endoplasmic reticulum;
RT	reverse transcriptase;
ORF	open reading frame;
RACE	rapid amplification of cDNA ends;
GSP	gene specific primer;
YPD	yeast peptone dextrose;
BMGY	buffered glycerol-complex;
BMMY	buffered methanol complex;
HPLC	high performance liquid chromatography.

#### **BRIEF DESCRIPTION OF THE DRAWINGS**

Fig. 1 illustrates the nucleotide and deduced amino acid sequence of the human  $\alpha$ 1,2-mannosidase cDNA;

Fig. 2 illustrates Northern blot analysis of human  $\alpha$ 1,2-mannosidase expression;

Fig. 3 illustrates the expression of the recombinant  $\alpha$ 1,2-mannosidase in *P. pastoris*;

Fig. 4 illustrates the time course human  $\alpha$ 1,2-mannosidase activity; and

Fig. 5 illustrates the  $[^1\text{H}]$ -NMR spectrum identifying the  $\text{Man}_8\text{GlcNAc}$  isomer produced by the human  $\alpha$ 1,2-mannosidase.

5

#### **DETAILED DESCRIPTION OF THE INVENTION**

In accordance with the present invention, there is provided the isolation of a novel human cDNA encoding a type II membrane protein of 79.5 kDa with amino acid sequence similarity to Class I  $\alpha$ 1,2-mannosidases. The catalytic domain of the enzyme was expressed as a  
10 secreted protein in *Pichia pastoris*. The recombinant enzyme removes a single mannose residue from  $\text{Man}_9\text{GlcNAc}$  and  $[^1\text{H}]$ -NMR analysis indicates that the only product is  $\text{Man}_8\text{GlcNAc}$  isomer B, the form lacking the middle-arm terminal  $\alpha$ 1,2-mannose. Calcium is required for enzyme  
15 activity and both 1-deoxymannojirimycin and kifunensine inhibit the human  $\alpha$ 1,2-mannosidase. The properties and specificity of this human  $\alpha$ 1,2-mannosidase are identical to the endoplasmic reticulum  $\alpha$ 1,2-mannosidase from *Saccharomyces cerevisiae* and differ from those of previously cloned mammalian (mouse and human) Golgi  $\alpha$ 1,2-  
20 mannosidases that remove up to four mannose residues from  $\text{Man}_9\text{GlcNAc}_2$  during N-glycan maturation. Northern blot analysis showed that all human tissues examined express variable amounts of a 3 kb transcript. This highly specific  $\alpha$ 1,2-mannosidase is likely to be involved in glycoprotein quality control since there is increasing evidence that  
25 trimming of  $\text{Man}_9\text{GlcNAc}_2$  to  $\text{Man}_8\text{GlcNAc}_2$  isomer B in yeast and mammalian cells is important to target misfolded glycoproteins for degradation. Furthermore, it has been shown that inhibition of this enzyme activity prevents the degradation of improperly folded mutant alpha1-antitrypsin characteristic of emphysema.

30

## Materials

Oligonucleotides were synthesized by BioCorp (Montréal, Canada). Man<sub>9</sub>GlcNAc was prepared from soya bean agglutinin and [<sup>3</sup>H]mannose-labeled Man<sub>9</sub>GlcNAc from rat liver as described previously (Jelinek-Kelly *et al.*, 1985; Bhattacharyya *et al.*, 1988). Man<sub>9</sub>GlcNAc isomer B substrate was prepared by digestion of Man<sub>9</sub>GlcNAc with the yeast  $\alpha$ 1,2-mannosidase. 1-deoxymannojirimycin, kifunensine and swainsonine were obtained from Toronto Research Chemicals, Inc. (Downsview, Canada). All other chemicals were reagent grade.

## Isolation of a novel human $\alpha$ 1,2-mannosidase cDNA

The NCBI dbEST database was searched with the yeast  $\alpha$ 1,2-mannosidase amino acid sequence (Camirand *et al.*, 1991) using the tBLASTn algorithm to identify novel  $\alpha$ 1,2-mannosidases. Retrieved ESTs were aligned using the DNASTAR SeqMan program (Madison, WI) and EST clones AA631254, R16652 and H46222 encoding the 3' region of the ORF were obtained from Genome Systems Inc. and sequenced. The following nested primers were designed based on the consensus sequence, 5'-CCACAGACCCAGCAAGGTGCC-3' and 5'-CTAGGCAGGGGTCCAGATAGG-3', and used to amplify clones containing additional 5' sequence from human placenta Marathon Ready cDNA (CLONTECH) according to the recommended protocol. A 5  $\mu$ l aliquot the PCR reactions was subcloned into pCRII using the original TA cloning kit (Invitrogen) and clones encoding  $\alpha$ 1,2-mannosidase were identified by hybridization of colony lifts with gene specific <sup>32</sup>P-labeled oligonucleotide probes. The longest clones (2.1 kb) were sequenced.

Thereafter the Gibco 5' RACE System (Version 2) was used to isolate additional 5' sequence. First strand cDNA was synthesized from 2.5  $\mu$ g of placenta, testis, and liver total RNA (CLONTECH) at 55°C using ThermoScript RT (Gibco) and a gene specific primer (GSP1 5'-GGGCACTTCTGCTCTTCTTGAAG-3') located within the 5' region of the

placenta cDNA clones. Nested 5' RACE amplicons were then generated using gene specific primers (GSP2 5'-ATGACTGTCCTCTGCGGATCTC-3', and GSP3.1 5'-TGTCTTCTGTGACGAAATCTC-3' or GSP3.2 5'-CAGAGCTTTCCAATGGTCAGC-3' or GSP3.3 5'-TCATAGCTCTCGCCA  
5 AAGCTCAGC-3') and Platinum *Taq* (Gibco) according to the recommended protocol. The amplicons were subcloned into pCR2.1 (Invitrogen), identified by hybridization of colony lifts with gene specific <sup>32</sup>P-labeled oligonucleotide probes, and sequenced. In addition, Genome  
10 Systems isolated three fetal brain  $\alpha$ 1,2-mannosidase cDNA clones (2.7 kb) by a PCR and hybridization cDNA library screen using primers located within the 5' ORF (5'-ATCGGGACTTCACCTCGGTG-3' and 5'-CAGAGC TTTCCAATGGTCAGC-3').

#### Northern blot analysis

15 The human  $\alpha$ 1,2-mannosidase EST R16652 was labeled with [ $\alpha$ -<sup>32</sup>P]dCTP (3000 Ci/mmol) using the multiprime DNA labeling kit (Amersham). The probe was hybridized to human multiple tissue Northern blots (CLONTECH) according to the recommended protocol and exposed to x-ray film for 5 days (Kodak).

#### 20 Expression of the catalytic domain in *Pichia pastoris*

The DNA sequence encoding the soluble catalytic domain of the  $\alpha$ 1,2-mannosidase was amplified from a cDNA clone by PCR using the sense oligonucleotide 5'-AAAGAATTCCAGATTAGACCCCCAAGCCA  
25 AG-3' containing an *Eco*RI site and the antisense primer SHMSTOP 5'-AAATCTAGACTAGGCAGGGGTCCAGATAGG-3' containing the stop codon followed by an *Xba*I site. Expression vector pZ $\alpha$ ASHM169 was constructed by ligation of the amplicon into the *Eco*RI/*Xba*I sites of pPICZ $\alpha$  A (Invitrogen) in frame with the  $\alpha$ -factor secretion signal. Similarly,  
30 shorter untagged (pZ $\alpha$ ASHM240) and tagged (pZ $\alpha$ ASHM240T) expression constructs were prepared using sense primer 5'-AAAGA



ATTCCAGGGCACACCAGTGCATCTG-3' and antisense primers SHMSTOP, and SHMR 5'AAATCTAGAGCAGGGGTCCAGATAGGCAG-3' respectively. Primer SHMR lacks a termination codon thus the C-terminal of the recombinant enzyme was fused to the (His)<sub>6</sub> and Myc tags encoded by pPICZα A.

*Pichia pastoris* strain GS115 (*his4*) (Invitrogen) was transformed by electroporation with 10 µg of the expression constructs linearized with Pmel. Transformants were grown and assayed for α1,2-mannosidase activity as described previously.

10

#### α-Mannosidase Assays

To characterize the recombinant α1,2-mannosidase, medium containing the enzyme was concentrated 7.5 fold using centrifugal filters (Millipore) and equilibrated in 40 mM PIPES pH 6.5. Two microlitres of the concentrated medium was incubated with [<sup>3</sup>H]mannose-labeled Man<sub>9</sub>GlcNAc at 37°C and the amount of released [<sup>3</sup>H]mannose was determined by the Con A/PEG precipitation method.

Divalent cation requirements were studied by including 0.05 mM EDTA in duplicate assays in the absence or presence of 2 mM CaCl<sub>2</sub>, ZnCl<sub>2</sub>, MnCl<sub>2</sub>, MgCl<sub>2</sub>, and CoCl<sub>2</sub>. The enzyme was incubated for 2 hours with 5000 cpm of [<sup>3</sup>H]Man<sub>9</sub>GlcNAc in 40 mM PIPES pH 6.5, 1 mg/ml BSA, and 1 mM NaN<sub>3</sub>.

The K<sub>m</sub> was determined by Lineweaver-Burk analysis. Duplicate 30 min assays contained Man<sub>9</sub>GlcNAc substrate (0.05-0.5 mM), 5000 cpm of [<sup>3</sup>H]Man<sub>9</sub>GlcNAc, 1 mM CaCl<sub>2</sub>, 40 mM PIPES pH 6.5, 1 mg/ml BSA, and 1 mM NaN<sub>3</sub>.

The effects of the Class I α-mannosidase inhibitors 1-deoxymannojirimycin and kifunensine, and the Class II α-mannosidase inhibitor swainsonine were investigated by preincubating the enzyme on ice for 30 min with the inhibitor in 40 mM PIPES pH 6.5, 1 mM CaCl<sub>2</sub>, 1 mg/ml BSA, and 1 mM NaN<sub>3</sub>. Substrate (0.8 mM Man<sub>9</sub>GlcNAc and 20,000

cpm of [ $^3\text{H}$ ]Man<sub>9</sub>GlcNAc) was then added to the duplicate assays and the mixtures were incubated at 37°C for 1 hour.

The time dependent formation of products was analyzed by assaying the recombinant enzyme at 37°C in a 30  $\mu\text{l}$  mixture containing  
5 3.4 mM Man<sub>9</sub>GlcNAc, 30,000 cpm [ $^3\text{H}$ ]Man<sub>9</sub>GlcNAc, 44 mM potassium phosphate pH 6.5, 1 mg/ml BSA, 1 mM NaN<sub>3</sub> and 12  $\mu\text{l}$  of unconcentrated medium. At 0, 1, 2, 4, 8, and 19.5 h one-sixth of the reaction mixture was collected. The products were resolved by HPLC, and identified by  
10 comparing their elution to that of the [ $^{14}\text{C}$ ]Glc<sub>3</sub>Man<sub>9</sub>GlcNAc internal standard, as described previously.

#### High Resolution [ $^1\text{H}$ ]-NMR Analysis

Medium containing the human  $\alpha$ 1,2-mannosidase was incubated at 37°C with 600  $\mu\text{g}$  Man<sub>9</sub>GlcNAc and 10<sup>5</sup> cpm [ $^3\text{H}$ ]Man<sub>9</sub>GlcNAc  
15 in 44 mM potassium phosphate buffer pH 6.5 containing 1 mg/ml BSA and 1 mM NaN<sub>3</sub>. The incubation was supplemented with additional enzyme after 8 and 22.5 h, and terminated after 28.5 h by boiling for 3 min. An aliquot of the sample was analyzed by HPLC to show that most of the sample was transformed to Man<sub>9</sub>GlcNAc. The sample was then  
20 chromatographed on a Bio-Gel P-6 column (1 X 109 cm) equilibrated in deionized water. Fractions containing the oligosaccharide product were pooled, lyophilized, resuspended in D<sub>2</sub>O and lyophilized four times, and stored over P<sub>2</sub>O<sub>5</sub> in a vacuum desiccator. The [ $^1\text{H}$ ]-NMR spectra were recorded at Université de Montréal NMR Facility in 5 mm tubes using a  
25 600 MHz Bruker spectrometer at 30 and 70°C with the acetone chemical shift set to 2.225 ppm with respect to 4,4-dimethyl-4-silapentane sulfonate.

#### Polyacrylamide gel electrophoresis Western blotting

SDS-PAGE was performed using the Bio-Rad Mini-Protean II  
30 apparatus as described by Laemmli. Western blots were prepared by transferring proteins onto a nitrocellulose membrane (Schleicher and

Schuell), and expression of the Myc-tagged recombinant  $\alpha$ 1,2-mannosidase was detected using the monoclonal Anti-myc Antibody (Invitrogen) and visualized by the ECL Western blotting detection system (Amersham).

5

## **DNA Sequencing and Alignments**

Manual sequencing was performed using the Pharmacia T7 and Deaza sequencing kits. The Sheldon Biotechnology Centre Automated Sequencing Facility (McGill University, Montréal, Canada) employed the  
10 ABI prism dye terminator and thermo sequenase fluorescent labeled primer cycle sequencing kits and the samples were run on the ABI 373A (Perkin Elmer) and ALFexpress (Amersham Pharmacia Biotech) sequencers respectively. The DNASTAR SeqMan program (Madison, WI) was used to assemble the sequences into contigs. The deduced amino  
15 acid sequences were aligned using the BestFit and Publish programs (Version 9.1) from the University of Wisconsin Genetics Computer Group (Madison, WI).

## **Isolation and characterization of a novel human $\alpha$ 1,2-mannosidase cDNA**

20 A novel human  $\alpha$ 1,2-mannosidase was identified by searching the EST database with the yeast  $\alpha$ 1,2-mannosidase amino acid sequence (Camirand *et al.*, 1991). The consensus sequence of the identified overlapping clones encodes the terminal 50 amino acids of the catalytic  
25 domain including two of the most highly conserved Class I  $\alpha$ -mannosidase sequence motifs followed by 589 bp of 3' UTR terminating in a poly A tail. Clones encoding the complete ORF (2.1 kb) and 5' UTR were then isolated by nested 5' RACE from human placenta, liver and testis cDNAs, as described in materials and methods. In addition an independent 2.7 kb  
30 clone containing the ORF flanked by 50 bp of 5' UTR and 589 bp of 3' UTR terminating with a polyA tail was isolated from a human fetal brain

cDNA library. The sequence of the cDNAs obtained from the different sources were identical.

The 2.7 kb cDNA is predicted to encode a 79.5 kDa type II membrane protein with an 85 amino acid cytoplasmic tail, followed by a putative transmembrane domain of about 17 residues, a "stem" region of about 137 amino acids not required for enzyme activity and a large C-terminal catalytic domain (amino acids 240-699) (Fig. 1). The 5' UTR and ORF sequence were obtained from human placenta, testis and liver cDNA clones amplified by RACE, and a fetal brain cDNA library clone. The 3' UTR sequence is the consensus of an alignment of EST clones. Bold numbers refer to the deduced amino acid sequence and the numbers in normal type refer to the nucleotide sequence. The conserved Class 1  $\alpha$ -mannosidase sequence motifs are underlined, and the highly conserved invariant acidic amino acid residues as well as the conserved cysteines common to all class I  $\alpha$ -mannosidases are boxed in grey. The putative transmembrane domain sequence is indicated in bold and underlined. Arrows indicate the starting amino acid residues of the different forms of the recombinant enzyme expressed in *Pichia pastoris*.

The cytoplasmic tail is much longer than any of the type II membrane-bound glycosidases or glycosyltransferases described so far, and contains a proline rich domain. The catalytic domain of this novel  $\alpha$ 1,2-mannosidase contains the highly conserved sequence motifs characteristic of Class I  $\alpha$ -mannosidases as well as the highly conserved disulfide bonded cysteines and acidic amino acid residues that are essential for enzymatic activity. The catalytic domain of the human  $\alpha$ 1,2-mannosidase is 43% identical (54% similar) to the yeast  $\alpha$ 1,2-mannosidase (Camirand *et al.*, 1991), and 40% identical (54% similar) to human and murine  $\alpha$ 1,2-mannosidase IA and IB. There is no significant similarity between the N-terminal sequence of this novel  $\alpha$ 1,2-mannosidase and previously cloned members of the same family.

Northern blot analysis indicates that all tissues examined expressed variable levels of a 3 kb transcript (Fig. 2). Random labeled  $\alpha$ 1,2-mannosidase EST clone R16652 was hybridized to Northern blots containing 2  $\mu$ g of poly (A<sup>+</sup>) RNA from human tissue. The blots were  
5 exposed to film to x-ray film for 5 days.

The expression is particularly high in testis and relatively low in lung and muscle. The gene encoding this novel  $\alpha$ 1,2-mannosidase is localized on chromosome 9 since a clone (T12605) isolated from a human chromosome 9 cosmid library contains exonic sequence identical to the  
10 cDNA sequence (nucleotides 731-916).

#### **Expression of recombinant $\alpha$ 1,2-mannosidase in *Pichia pastoris***

The  $\alpha$ 1,2-mannosidase was expressed as a secreted protein in *P.pastoris* in order to characterize its enzymatic activity. The catalytic  
15 domain starting at either amino acid 169 or 240 was cloned in frame downstream from the  $\alpha$ -factor of the *P.pastoris* expression vector pPICZ $\alpha$  A. Following methanol induction of yeast transformed with expression constructs pZ $\alpha$ ASHM169, pZ $\alpha$ ASHM240 or pZ $\alpha$ ASHM240T similar levels of  $\alpha$ 1,2-mannosidase activity were detected in the medium, and absent in  
20 yeast transformed with the pPICZ $\alpha$  A vector. Recombinant  $\alpha$ 1,2-mannosidase of the expected size (55 kDa) was observed by Western blot analysis at 2-3 days post-induction and decreased thereafter (Fig. 3). Ten microlitres of medium was subjected to reducing SDS-PAGE (10%) and the recombinant Myc tagged  $\alpha$ 1,2-mannosidase was visualized by  
25 Western blot analysis. Lanes 1-4 correspond to GS115 transformed with pZ $\alpha$ ASHM240T at 2, 3, 4, and 5 days post-induction, respectively. Lane 5 corresponds to GS115 transformed with vector at 5 days post-induction. Molecular mass standards are indicated on the right.

### Properties of the human $\alpha$ 1,2-mannosidase

The catalytic properties of the recombinant  $\alpha$ 1,2-mannosidase were studied using [ $^3$ H]Man $_5$ GlcNAc as substrate. The enzyme is active over a pH range of 6.3-7.2 with an optimum between 6.5-6.9. Inclusion of at least 0.1 mM Ca $^{2+}$  in the assay is required to obtain maximum enzyme activity. The enzyme is inhibited 50% by 1  $\mu$ M EDTA and 100% by 25  $\mu$ M EDTA. This inhibition is completely reversed by the addition of 2 mM Ca $^{2+}$  but, not by 2 mM Mn $^{2+}$ , Mg $^{2+}$ , Zn $^{2+}$  or Co $^{2+}$ . The  $K_m$  of the recombinant enzyme is 0.4 mM which is similar that of the recombinant yeast  $\alpha$ 1,2-mannosidase (0.3 mM).

The human  $\alpha$ 1,2-mannosidase is inhibited by the Class I  $\alpha$ -mannosidase inhibitors, 1-deoxymannojirimycin (IC $_{50}$  = 75  $\mu$ M) and kifunensine (IC $_{50}$  = 70 nM), but is insensitive to the Class II  $\alpha$ -mannosidase inhibitor swainsonine (Table I).

**Table I**

Effect of  $\alpha$ -mannosidase inhibitors on the human  $\alpha$ 1,2-mannosidase activity

Inhibitor	Activity <sup>a</sup> (% of control)
1-deoxymannojirimycin	
10 $\mu$ M	84
50 $\mu$ M	62
500 $\mu$ M	10
kifunensine	
0.05 $\mu$ M	67
0.10 $\mu$ M	28
0.50 $\mu$ M	0
swainsonine	
5 $\mu$ M	100
10 $\mu$ M	100
100 $\mu$ M	100

<sup>a</sup> Assay conditions are described in materials and methods. Activity is expressed as a percentage of [ $^3$ H]mannose released in the absence of inhibitors (226 dpm).

### Specificity of the human $\alpha$ 1,2-mannosidase

The recombinant  $\alpha$ 1,2-mannosidase was incubated with [ $^3$ H]Man<sub>8</sub>GlcNAc for different periods of time and HPLC analysis showed that the only products formed are Man<sub>8</sub>GlcNAc and mannose in a time dependent manner (Fig. 4). [ $^3$ H]Man<sub>8</sub>GlcNAc was incubated with medium obtained from *P.pastoris* transformed with pZ $\alpha$ ASHM169 two days post-induction. Oligosaccharide product formation was monitored by HPLC as described in materials and methods and is expressed as a percentage of the total radioactivity recovered.

Supplementing the incubation mixture with fresh enzyme after 8 and 22.5 hours of incubation did not result in any further mannose trimming. The Man<sub>8</sub>GlcNAc oligosaccharide formed was demonstrated to be isomer B by [ $^1$ H]-NMR analysis (Fig. 5). Spectrum at 600 MHz and 30°C of the anomeric region of the Man<sub>8</sub>GlcNAc produced by the hydrolysis of Man<sub>8</sub>GlcNAc by the specific human  $\alpha$ 1,2-mannosidase. The resonance corresponding to the anomeric proton of each mannose residue is numbered. Integral values for each of the anomeric protons, except GlcNAc, were obtained. The split resonance signal at 5.104 and 5.075 ppm for residue 7 is characteristic of Man<sub>8</sub>GlcNAc isomer B.

Consistent with the strict specificity of this enzyme, Man<sub>8</sub>GlcNAc isomer B and p-nitrophenyl- $\alpha$ -D-mannopyranoside are not substrates of the enzyme. Therefore this highly specific  $\alpha$ 1,2-mannosidase is the human ortholog of the yeast processing  $\alpha$ 1,2-mannosidase that has been implicated in the targeting of misfolded glycoproteins for degradation (Knop *et al.*, 1996; Jakob *et al.*, 1998). Since there is evidence that *N*-glycan trimming by 1-deoxymannojirimycin and kifunensine-sensitive ER  $\alpha$ 1,2-mannosidase activity is also implicated in the degradation of misfolded glycoproteins in mammalian cells (Su *et al.*, 1993; Liu *et al.*, 1997; Yang *et al.*, 1998; Liu *et al.*, 1999), it seems likely that the novel

human  $\alpha$ 1,2-mannosidase we have cloned is involved in ER quality control, but this remains to be shown.

The present invention will be more readily understood by referring to the following examples which are given to illustrate the invention rather than to limit its scope.

### **EXAMPLE I**

Possible approaches whereby cloning and expression of this new human  $\alpha$ 1,2-mannosidase can be used to design new specific inhibitors that might be developed for drug therapy of glycoprotein folding diseases:

- (1) use of recombinant enzyme for high throughput screening of naturally occurring compounds as potential specific inhibitors.
- (2) use of recombinant enzyme to test chemically synthesized derivatives of mannodeoxynojirimycin and kifunensine as potential specific inhibitors.
- (3) molecular modelling of the human mannosidase based on its amino acid sequence and the recently determined three-dimensional structure and identification of the active site of the yeast  $\alpha$ 1,2-mannosidase ortholog that has the same properties, specificity and function in degradation of misfolded glycoproteins.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.



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**WHAT IS CLAIMED IS:**

1. A human  $\alpha$ 1,2-mannosidase enzyme for specifically converting  $\text{Man}_9\text{GlcNAc}$  to  $\text{Man}_8\text{GlcNAc}$  isomer B in degradation mechanism of misfolded proteins, wherein said enzyme has the characteristics of an enzyme encoded by a cDNA sequence set forth in Fig. 1.
  2. An agonist or antagonist of the  $\alpha$ 1,2-mannosidase enzyme of claim 1 for activating or inhibiting said enzyme.
  3. The agonist or antagonist of claim 2, wherein said activating or inhibiting is for a transient period of time.
  4. An antagonist of claim 2, wherein said inhibiting is for a transient period of time, thereby preventing degradation of misfolded glycoproteins.
  5. A method for the treatment of a genetic disease causing a misfolding of proteins in a patient, which comprises administering an antagonist of  $\alpha$ 1,2-mannosidase enzyme of claim 1 for transiently inhibiting said enzyme, thereby preventing degradation of misfolded glycoproteins.
  6. The method of claim 5, wherein said genetic disease is selected from the group consisting of cystic fibrosis and emphysema.
  7. The method of claim 6, wherein for cystic fibrosis said misfolded protein is cystic fibrosis transmembrane conductance regulator (CFTR).
  8. The method of claim 6, wherein for emphysema said misfolded protein is alpha1 antitrypsin.
-

**ABSTRACT OF THE INVENTION**

The present invention also relates to the use of the recombinant  $\alpha$ 1,2-mannosidase for the development of specific agonist or antagonist of the specific  $\alpha$ 1,2-mannosidase enzyme for specifically converting  $\text{Man}_9\text{GlcNAc}$  to  $\text{Man}_9\text{GlcNAc}$  isomer B in degradation mechanism of misfolded proteins, wherein the enzyme has the characteristics of an enzyme encoded by a cDNA sequence set forth in Fig. 1. The present invention also relates to an agonist or antagonist of the  $\alpha$ 1,2-mannosidase enzyme of claim 1 for activating or inhibiting the enzyme. The present invention also relates to a method for the treatment of genetic diseases resulting in misfolding of glycoproteins in a patient, which comprises administering an antagonist of  $\alpha$ 1,2-mannosidase enzyme of claim 1 for transiently inhibiting the enzyme, thereby stabilizing misfolded glycoproteins and preventing their degradation.

Applicant or Patentee: Annette A. HERSCOVICS et al.  
Serial or Patent No.: \_\_\_\_\_ Atty. Dkt. No.: 1770-228"USPR" FC/ld  
Filed or Issued: \_\_\_\_\_  
For:  $\alpha$ 1,2-MANNOSIDASE AND THERAPEUTICAL USES THEREOF

**VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS**  
**[(37 CFR 1.9(f) AND 1.27 (d)) - NONPROFIT ORGANIZATION]**

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

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(CITATION OF STATUTE \_\_\_\_\_)

I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 CFR 1.9 (e) for purposes of paying reduced fees under section 41(a) or (b) of Title 35, United States Code with regard to the invention entitled  $\alpha$ 1,2-MANNOSIDASE AND THERAPEUTICAL USES THEREOF by inventor(s) Annette A. HERSCOVICS et al. described in

☒ (X) the specification filed herewith

☐ ( ) application serial no. \_\_\_\_\_, filed \_\_\_\_\_

☐ ( ) patent no. \_\_\_\_\_, issued \_\_\_\_\_

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above-identified invention.

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NAME OF PERSON SIGNING Alex Navarre

TITLE IN ORGANIZATION Director - Office of Technology Transfer

ADDRESS OF PERSON SIGNING 845 Sherbrooke Street West, Montréal, Québec, Canada H3A 2T5

SIGNATURE \_\_\_\_\_ DATE \_\_\_\_\_



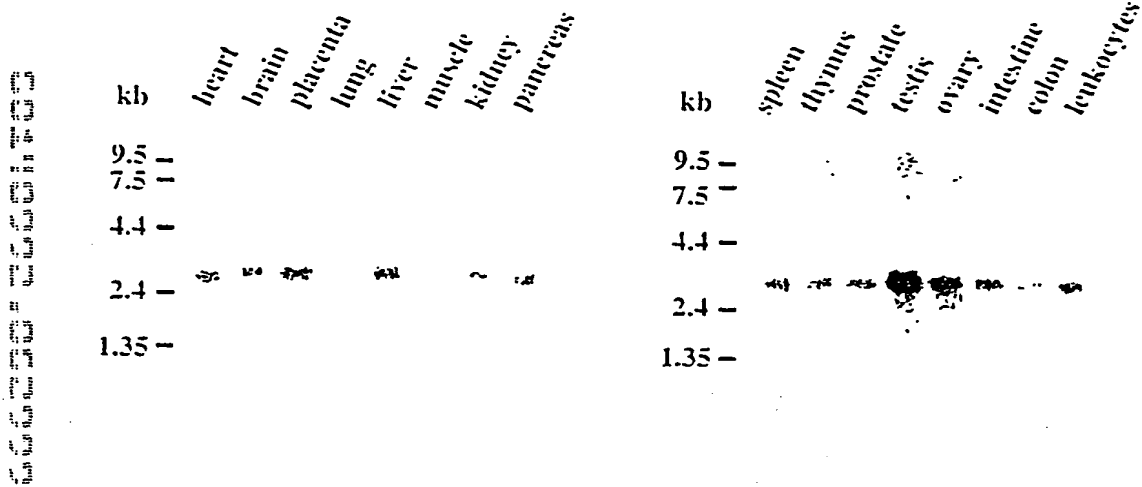


Fig. 2

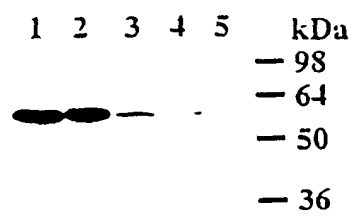


Fig. 3

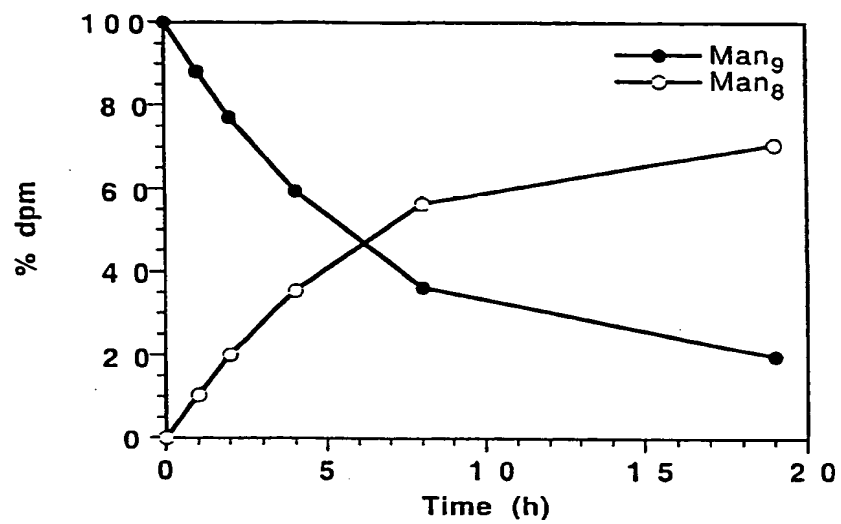


Fig. 4



5.4 5.3 5.2 5.1 5.0 4.9 4.8

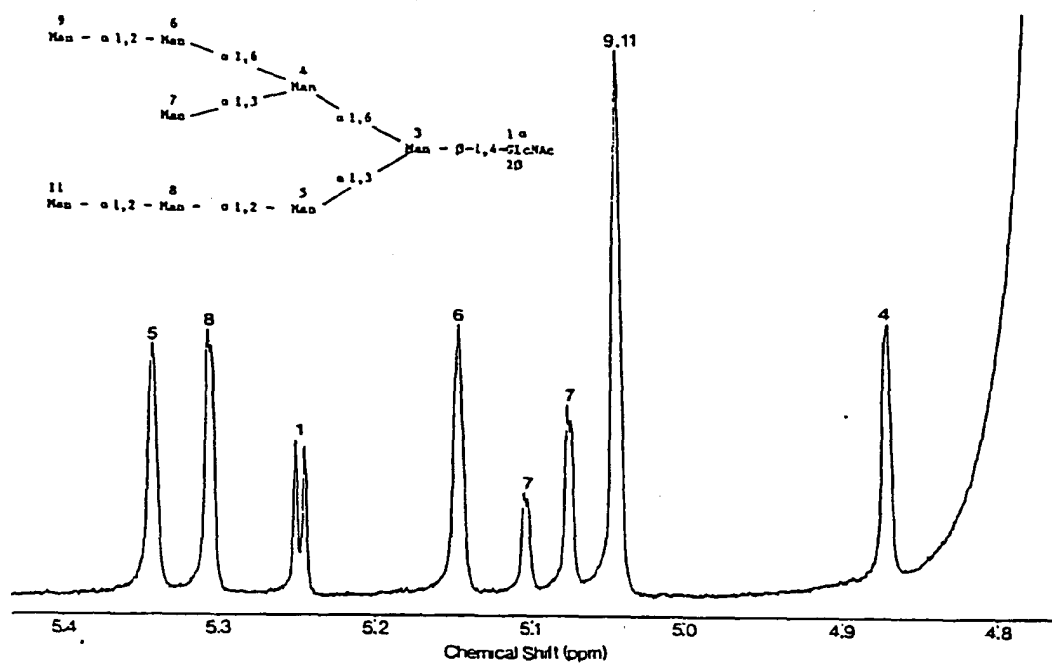


Fig. 5

